



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>G01N 33/66, 33/86</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/15406</b>  <b>(43) International Publication Date:</b> <b>5 August 1993 (05.08.93)</b>
<b>(21) International Application Number:</b> PCT/GB93/00197 <b>(22) International Filing Date:</b> 29 January 1993 (29.01.93)  <b>(30) Priority data:</b> 9202019.7 30 January 1992 (30.01.92) GB  <b>(71) Applicant (for all designated States except US):</b> IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE[GB/GB]; Exhibition Road, London SW7 2AZ (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> CASS, Anthony, Edward, George [GB/GB]; 108 Fernlea Road, Balham, London SW12 9RW (GB). SOHANPAL, Kalvinder [GB/GB]; Flat 5, Mulgrave Hall, Mulgrave Road, Sutton, Surrey SM2 6LG (GB).		<b>(74) Agent:</b> MARCH, Gary, Clifford; Batchellor, Kirk & Co., 2 Pear Tree Court, Farringdon Road, London EC1R 0DS (GB).  <b>(81) Designated States:</b> AU, CA, GB, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.            Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHODS AND APPARATUS FOR ASSAY OF SULPHATED POLYSACCHARIDES  <b>(57) Abstract</b>  <p>A method of assaying a sample for sulphated polysaccharide which comprises contacting the sample with (i) a complementary binding polymer capable of binding to said polysaccharide, said binding polymer being labelled with (ii) at least one optically active reporter group, said polymer (i) capable of forming (iii) a complex with said polysaccharide, the reporter group being sensitive to the formation and/or pressure of said complex, and subsequently measuring change in an optical property of the optically labelled polymer as a result of such contact, comparing the change in said optical property with values thereof derived from a control assay for the same polysaccharide, binding polymer and reporter group, and using the comparison of values between the sample and control assays to determine concentration of polysaccharide in said sample. The preferred polysaccharide is heparin, the complementary binding polymer is preferably polycationic polypeptide and the optical property is preferably fluorescence. Assays carried out by the present method can be quicker than previous such assays and may even be appropriate for <i>in vivo</i> use.</p>		

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METHODS AND APPARATUS FOR ASSAY OF SULPHATED POLYSACCHARIDES

The present invention is concerned with detection techniques, apparatus and probes for the measurement of sulphated polysaccharides and especially heparin. Heparin is a sulphated polysaccharide that is a potentiator of the anticoagulant activity of anti thrombin III, a naturally occurring anticoagulant present in the blood. Heparin is widely used in surgery to prevent blood coagulation during major operative procedures; the monitoring of its levels during the operation and appropriate adjustment of dose prevents premature coagulation during surgery.

Monitoring of heparin levels currently involves measurement of coagulation times in a standard serum preparation.

Several disadvantages include:

1. Subjectivity in assessing the onset of coagulation,
2. Variability in the nature of the serum standard, and
3. Measurement of the activity, rather than the quantity of heparin present.

Although measurement of coagulation times is the commonest method of assaying heparin others have been proposed in the literature. None of the systems in current use however are rapid, or appropriate for in vivo use.

US-A-4911549 (KARKAR) discloses use of a dye binding assay based on the shift in absorbance maximum of the dye Azure A when it is bound to heparin. This involves extracorporeal measurement and relatively complex apparatus which is unsuitable for in vivo use and needs relatively high fluid volumes. European Patent Application EP-A-0362077 (SANOFI) is concerned with chemical determination of heparin by absorption on a cationic material and addition of anionic indicator, removing excess material and relating values to the quantity of conjugated developer.

Heparin is a polyanionic material and binds to many proteins such as antithrombin III, lipoprotein lipase, histidine rich glycoprotein, laminin and collagen. Many of these interactions are responsible for heparin's physiological functions. It will also bind to antiheparin antibodies and heparinase as well as synthetic homo- and hetero-polypeptides. The present invention is based on measuring heparin by the interaction between heparin and a complementary binding polymer (CBP) such as a protein, the interaction between the two molecules causing physiochemical change in the properties of a reporter group attached to the CBP. In the reporter group, binding of heparin to the CBP can alter an optical property such as fluorescence.

According to this invention, there is provided in one aspect a method of performing an assay on a sample which may or does contain a sulphated polysaccharide of interest

which comprises contacting the sample with (i) a complementary binding polymer (CBP) which is labelled with (ii) at least one optically active reporter group and which polymer is capable of forming (iii) a complex with said polysaccharide, said reporter group being sensitive to formation and/or presence of said complex, followed by measuring change in an optical property of the optically-labelled polymer as a result of such contact, and comparing the change with values derived from a control assay for the same polysaccharide, polymer and reporter group and thereby to determine the concentration of the polysaccharide of interest in the sample.

In another aspect the invention provides means for carrying into effect the method of the first aspect including:

- (i) a complementary binding polymer (CBP) for the polysaccharide under evaluation,
- (ii) the polymer (i) being labelled with at least one optically active reporter group as specified hereinabove, and capable of forming
- (iii) a complex as specified above, the means further comprising
- (iv) instrumentation capable of measuring changes in the optical properties of the labelled polymer, and
- (v) the results of a known assay for the same polysaccharide, polymer and reporter group.

It is preferred to include as part of the means for conducting the assay, data processing means set up to reduce the results of an assay to a more easily readable form, more preferably capable of displaying the concentration of the polysaccharide (such as heparin) in 'real time' as the or each further assay is conducted. This is particularly so for in vivo use where it is most desirable to know the actual instantaneous heparin concentration at any given time during an operative surgical or other medical procedure.

It is also preferred to provide an insoluble support for the optically labelled, complex-forming binding polymer. Such support is more preferably immobilised and may form a component part of heparin concentration detection means, such as an optical probe attached to optical fibre upon which alteration in the optical property, notably quenching of fluorescence, can be monitored.

For embodiments of the invention which employ an immobilised support, such as a solid, unreactive surface or polymer, that support may comprise or be part of an electrode, probe or optical waveguide.

In preferred embodiments the binding of heparin to a synthetic polycationic peptide or other polymer is measured by the change in the fluorescence intensity of a 'fluorophore' group attached to the polymer. In the specific embodiments described the polymer is either poly(L-ornithine), poly(L-lysine) or poly(ethyleneimine) although

other examples are possible.

The fluorescence reporter group may be, for example either fluorescein or tetramethylrhodamine although other types of fluorescent group might behave similarly. Preferably the fluorescent group is attached to the CBP through the latter's amino groups (in the case of polypeptide) although other chemical linkages may be employed. The ratio of fluorescent group to monomer unit can be varied to optimise the response range of the assay.

In the embodiments described herein, quenching of fluorescence intensity is measured to monitor the reaction.

In a more preferred embodiment the reaction between the CBP and heparin occurs on a solid surface which also acts to guide in the excitation light from a source and to carry the emitted light to a detector. However other configurations are possible including those where the reaction is carried out on a solid surface attached to the optical system and the fluorescence is subsequently measured or those where the solid surface is placed in a fluorescence spectrometer or those where the complex formation between heparin and the labelled CBP is carried out in solution and the fluorescence properties of the solution are subsequently measured.

It is preferred that the CBP, as defined herein, have polycationic domains which are capable of complex formation with the anionic domains of the sulphated

polysaccharide. The most preferred polysaccharide is heparin. The polymer selected may have various molecular weights, and the dose-response curve may be dependent on the molecular weight. The CBP is also preferably hydrophilic. It is preferred to select a complementary binding polymer which is bio-compatible and which might be effective and thus suitable for in vivo use.

Fluorescence as the optical property is preferred, and the fluorescent label (fluorophore) preferably exhibits fluorescence in the visible to near-infrared wavelength range. For such purpose commercially available fluorophores as demonstrated in the figures may be deployed. The complementary binding polymer should preferably only be chemically reactive in the sense that it can be fluorescently labelled and bind ionically to the polysaccharide, preferably a strongly anionic polysaccharide such as heparin. The fluorescent label selected should still fluoresce when bound to the said polymer.

As far as the assays are concerned, there should be a quantum change in the optical property which is capable of detection and quantitative measurement. The property can increase or decrease in the presence of the polysaccharide of interest, although with heparin it appears quenching of fluorescent activity is sensitive to increase in heparin concentration. In order to determine whether any given polymer and fluorophore may be suitable for use in any



aspect of the present invention, the notional skilled worker need only conduct simple laboratory experiments. In short, if a detectable change occurs in the optical property of the optically active reporter group in response to variation in e.g. heparin concentration, then that reporter group and polymer are potentially usable.

Means for assaying heparin in accordance with the invention may be supplied in kit form, in which case the kit may additionally comprise a solid surface to which the CBP would bind e.g. polystyrene microtitre plates, polystyrene or polyamide film for external analysis or probe means comprising an immobilised support for binding purposes wherein there is preferably covalent linkage between the immobilised support and the labelled CBP.

A light source will be needed for photometric quantitative determinations preferably incorporating means for selecting a small band of wavelength of maximum absorption of the label in use, coupled with means for applying light to the labelled polymer such as a lens, an air gap or an optical fibre. Additionally it may be necessary to incorporate means, such as filter means, to select the wavelength of light emitted from the sample under investigation, means such as an optical fibre to direct emitted light to a photodetector and means such as a photodiode or photomultiplier tube to detect the intensity of the emitted light from the labelled polymer. Apparatus

for such purposes is shown schematically in Figures 2a and 2b .

For example, readings of light intensity can be taken via a Perkin Elmer fluorimeter LS 50 coupled with data processing means which store and interpret previously assayed standards - calibration curves which plot, for the CBP - fluorophore in use, intensity of emitted light against concentration of heparin over the therapeutic range. A baseline reading with no heparin present may be first taken and other readings taken which may be specified as a percentage change. Sensitivity and accuracy of the readings can be influenced by selection of (a) the fluorophore, (b) the ratio of fluorophore groups to repeating monomer units and (c) the nature of the polymer, e.g. protein, selected as the CBP.

In order that the invention may be more readily appreciated and carried into effect, the application of these principles to the measurement of heparin is now described with reference to the accompanying figures, wherein:

Figure 1 shows the molecular structures of some possible polycationic CBPs,

Figure 2 shows the molecular structures of some 'fluorophores' that can be used to label the polycations shown in Figure 1, or other suitable polycationic molecules,

Figure 2a shows one form of optical fibre

fluorimeter including movable probe as an embodiment of a heparin concentration detector,

Figure 2b is a schematic view of components suitable for conducting the assays on a fixed, immobilised support plate,

Figures 3a and 3b show fluorescence emission spectra of some the reporter groups of Figure 2 attached to some of the CBPs of Figure 1, whereby

Figure 3a shows the fluorescence emission spectra of fluorescein isothiocyanate (FITC) bound to poly-L-ornithine, using an excitation wavelength of 495nm and scanning the emission from 500-550nm.

Figure 3b shows the fluorescence emission spectra of tetramethylrhodamine isothiocyanate bound to poly-L-ornithine as a polymer conjugate using an excitation wavelength of 550nm and scanning the emission from 560-600nm,

Figure 4 shows the quenching effect of heparin upon the fluorescence intensity, (at 520nm of the fluorophore (FITC) attached to different molecular weight polymer of poly-L-lysine. (PLA = 10,700, PLB = 64,800 and PLC = 205,000). Concentration of poly-L-lysine in test sample was approx. 95µg/ml,

Figure 5 shows the quenching effect of heparin upon the fluorescence emission intensity, (at 525nm) of FITC attached to different m.w polymers of poly (ethyleneimine) - EthA =

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50-60,000, EthB = 1,200, EthC = 18,200. Concentration of polymer in test solution was approx. 100µg/ml, Figure 6 - shows the quenching effect of heparin upon the fluorescence emission intensity (at 577nm), of tetramethylrhodamine isothiocyanate (TRITC) attached to different m.w polymers of poly(ethyleneimine). Concentration of polymer in test sample was approx. 100µg/ml,

Figure 7 shows how the range of the standard curve can be manipulated by using different concentrations of polymer (ORAA = 100µg/ml, ORAB = 200µg/ml, ORAC = 300µg/ml) and label (label concentration for ORAA = 0.75mg/ml, ORAB = 0.50mg/ml, and for ORAC = 0.25mg/ml). The polymer used in this study was poly-L-ornithine (m.wt 19,900) and the fluorophore was FITC, and

Figure 8 shows the effect of heparin on the fluorescence emission intensity of TRITC bound to poly-L-ornithine (m.wt 19,900, conc 200µg/ml) and demonstrates how the curve can be adjusted by changing the label concentration (Label conc for A = 1mg/ml B = 0.75mg/ml, C = 0.50mg/ml and D = 0.25mg/ml).

In Figure 1 the repeating units of polyornithine (1) are shown with its cationic amino domains and similarly for polyethyleneimine (2) both of which are capable of binding to heparin to form a complex.

Figure 2 shows labelling of binding polymer (4) with fluorescein isothiocyanate (3) FITC to form fluorescently labelled polymer (5) and labelling of binding polymer (4) with tetramethylrhodamine isothiocyanate (6) TRITC to form the labelled polymer (7).

Figure 2a shows one embodiment of a fibre fluorimeter in which three optical fibres 1 are linked via beam splitter 2. The fibre end 3 shows an expanded view with a binding protein immobilised thereon. Light from a

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light source 5 passes through excitation filter 4 to the fibre end 3, and emitted light passes through emission filter 6 to a photodetector 7. The end 3 can be a probe for in vivo use.

An alternative arrangement in Figure 2b shows a wave guide 3b with binding protein immobilised on a solid surface thereof. Light from light source 5 causes excitation indicated by arrow 8, the binding protein fluoresces indicated by arrow 9, which is measured by photodetector 7 after passing through emission filter 6.

All measurements being carried out in human serum, Figure 4 shows the effect of heparin on the fluorescence emission intensity of a fluorescein group bound to poly(L-lysine) of differing molecular weights and illustrates how, by choice

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of the appropriate CBP, the dose-response curve can be adjusted for different concentration ranges.

Figure 5 shows the effect of heparin on the fluorescence emission intensity of a fluorescein group bound to poly(ethyleneimine) of differing molecular weights and illustrates how, by choice of the appropriate CBP, the dose-response curve can be adjusted for different concentration ranges.

Figure 6 shows the effect of heparin on the fluorescence emission intensity of a tetramethylrhodamine group bound to poly(ethyleneimine) of differing molecular weights and illustrates how, by choice of the appropriate CBP, the dose-response curve can be adjusted for different concentration ranges.

Figure 7 shows the effect of heparin on the fluorescence emission intensity of a fluorescein group bound to poly(L-ornithine) of differing degrees of labelling and illustrates how, by choice of the appropriate labelling conditions, the dose-response curve can be adjusted for different concentration ranges.

Figure 8 shows the effect of heparin on the fluorescence emission intensity of a tetramethylrhodamine group bound to poly(L-ornithine) of differing degrees of labelling and illustrates how, by choice of the appropriate labelling conditions, the dose-response curve can be adjusted for different concentration ranges.

The data from any of Figures 3a to 8 provide a quantitative relationship between fluorescence alteration and heparin level. As such the data can be stored as a known, control assay in e.g. a computerised database on suitable hardware and (software) including a monitor or other display for reading off the heparin level, using parameters derived from the given fluorescent label in use. Less preferably evaluation and determination of the heparin assay could be effected manually, by using the appropriate graph as a calibrating guide.

With reference to the above figures it can be seen that the dose-response curve for heparin can be tuned over a wide range by appropriate adjustment of the following properties:

1. Type of monomer unit in the polycation.
2. Molecular weight of the polycation.
3. Nature of the fluorescent reporter group.
4. Degree of labelling of the polycation.

Further factors influencing the choice of labelled CBP include those designed to minimise interference from intrinsic blood constituents, to improve the stability of the material, to aid the attachment to a solid surface.

The present invention has advantages over the currently used systems in that the detection apparatus and method can be used for in vivo testing of heparin level in a suitably miniaturised form e.g. by using a probe comprising

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immobilised support, and a continuous "real time" display of actual heparin concentration. Furthermore the apparatus can be relatively simple in mechanical and optical construction, and may not require use by highly skilled operatives.



CLAIMS

1. A method of assaying a sample for sulphated polysaccharide which comprises contacting the sample with  
(i) a complementary binding polymer capable of binding to said polysaccharide, said binding polymer being labelled with (ii) at least one optically active reporter group, said polymer (i) capable of forming (iii) a complex with said polysaccharide, the reporter group being sensitive to the formation and/or presence of said complex, and subsequently measuring change in an optical property of the optically labelled polymer as a result of such contact, comparing the change in said optical property with values thereof derived from a control assay for the same polysaccharide, binding polymer and reporter group, and using the comparison of values between the sample and control assays to determine concentration of polysaccharide in said sample.
2. A method as claimed in claim 1 wherein the sulphated polysaccharide is polyanionic and capable of binding to at least one protein to form a complex.
3. A method as claimed in claim 2 wherein the polysaccharide comprises heparin.
4. A method as claimed in any preceding claim in which the polymer comprises at least one cationic  $\text{NH}_3$  group.
5. A method as claimed in claim 4 in which the polymer comprises polymer having repeating L-ornithine, L-lysine or ethyleneimine groups.
6. A method as claimed in any preceding claim wherein the reporter group is a fluorophore, the optical property is fluorescence, and the optical change is quenching thereof.

7. A method as claimed in any preceding claim in which the binding polymer is provided on an immobilised support which is part of an electrode, probe or optical waveguide.
8. Assay means, suitable for carrying into effect a method as claimed in any preceding claim, which comprises:  
complementary binding polymer capable of binding to the polysaccharide being assayed, said polymer being labelled with at least one optically active reporter group and the polymer capable of forming a complex with said polysaccharide,  
apparatus capable of measuring change in the optical properties of said labelled polymer, and  
information relating to a known control assay for the same polysaccharide, polymer and reporter group.
9. Assay means as claimed in claim 9 wherein the polysaccharide is as defined in claim 1 or 2 and/or the polymer is as defined in any of claims 4, 5 or 7.
10. Assay means as claimed in claim 8 or 9, in which the reporter group is a fluorophore, the optical property is fluorescence and the optical change is variation in fluorescence intensity.
11. Assay means as claimed in any one of claims 8 to 10 including data processing means adapted to display or print instantaneous concentration of polysaccharide as the assay proceeds.
12. Assay means as claimed in any one of claims 8 to 11 for heparin, utilising a polycationic, complex-forming complementary binding polymer, which is fluorescently labelled and immobilised on a suitable support.
13. Assay means as claimed in claim 12 in which the reporter group comprises fluorescein and/or

tetramethylrhodamine.

14. Assay means as claimed in claim 12 or 13 wherein the apparatus for measuring change incorporates a light source, an optical fibre or waveguide as a solid support upon which the complementary binding polymer is immobilised, and a photodetector adapted to detect variation in the fluorescence intensity of the polymer in use in the assay, for example such apparatus comprising a fluorimeter coupled with data processing means which store and interpret previously assayed controls.

15. Assay means as claimed in any one of claims 8 to 14 wherein the binding polymer is a bio-compatible, hydrophilic polypeptide.

16. Assay means as claimed in any one of claims 8 to 15 in kit form including at least one member having a solid surface to which the binding polymer can be attached.

17. Assay means as claimed in claim 16 wherein the member comprises a microtitre plate, a film or probe means coupled to spectrophotometer and concentration display means.

18. Use of assay means as claimed in any one of claims 8 to 17 for performing an assay according to a method as claimed in any one of claims 1 to 7.

FIG. 1

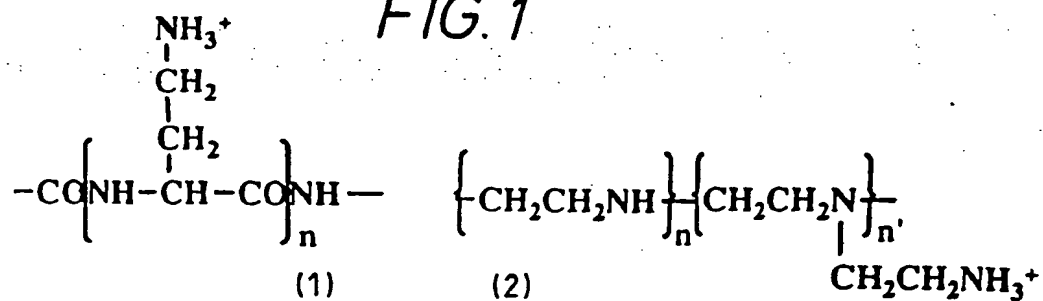
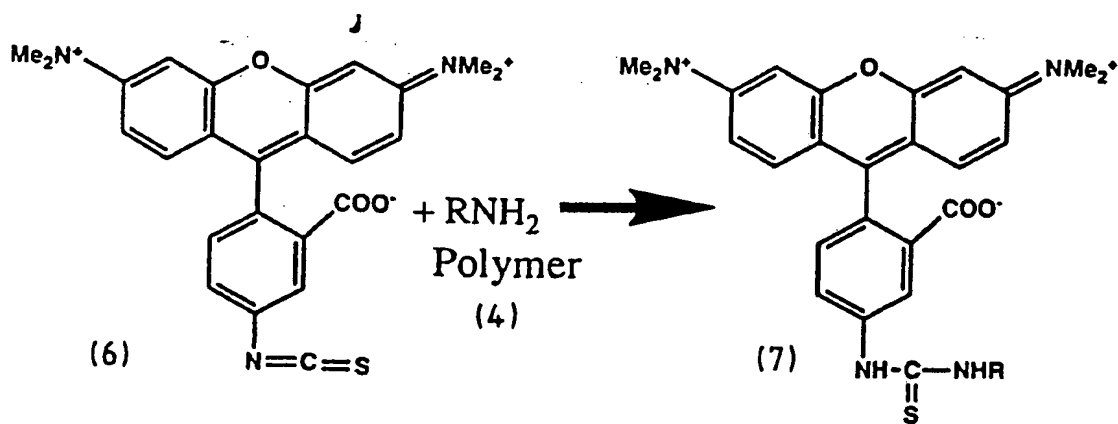
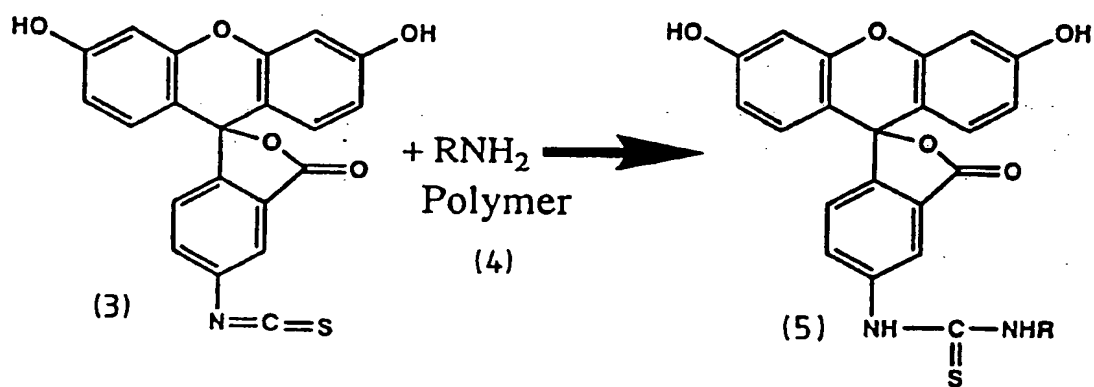


FIG. 2



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FIG. 2a

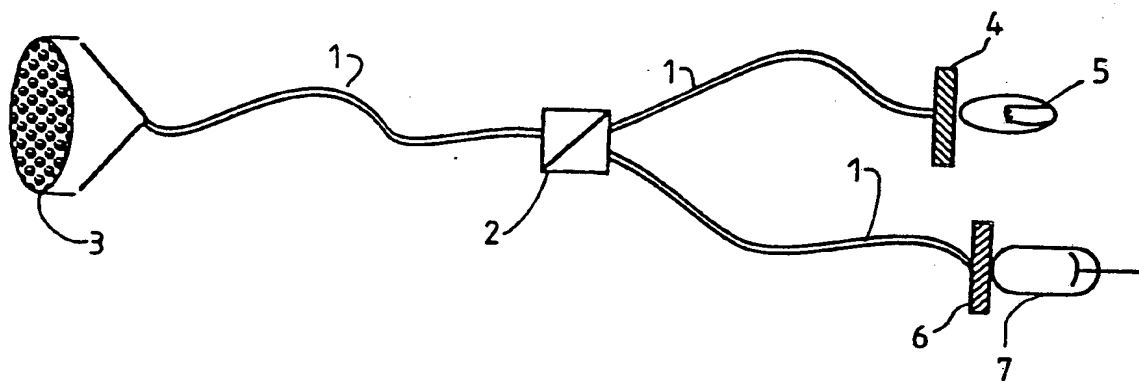
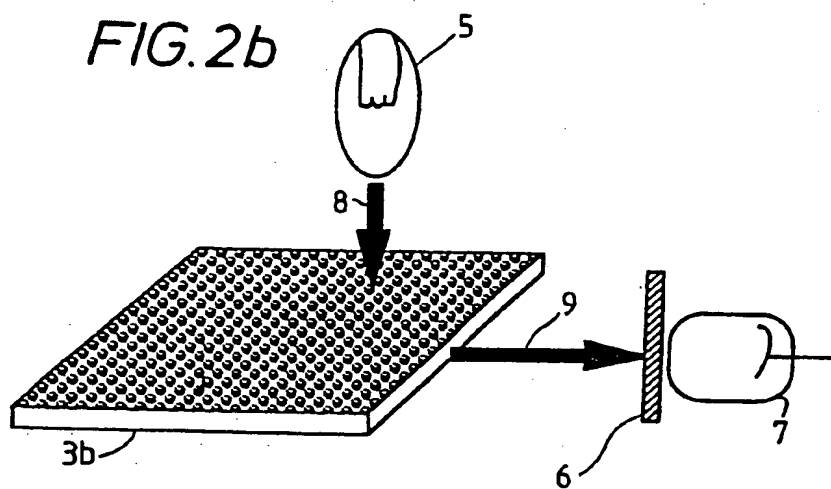
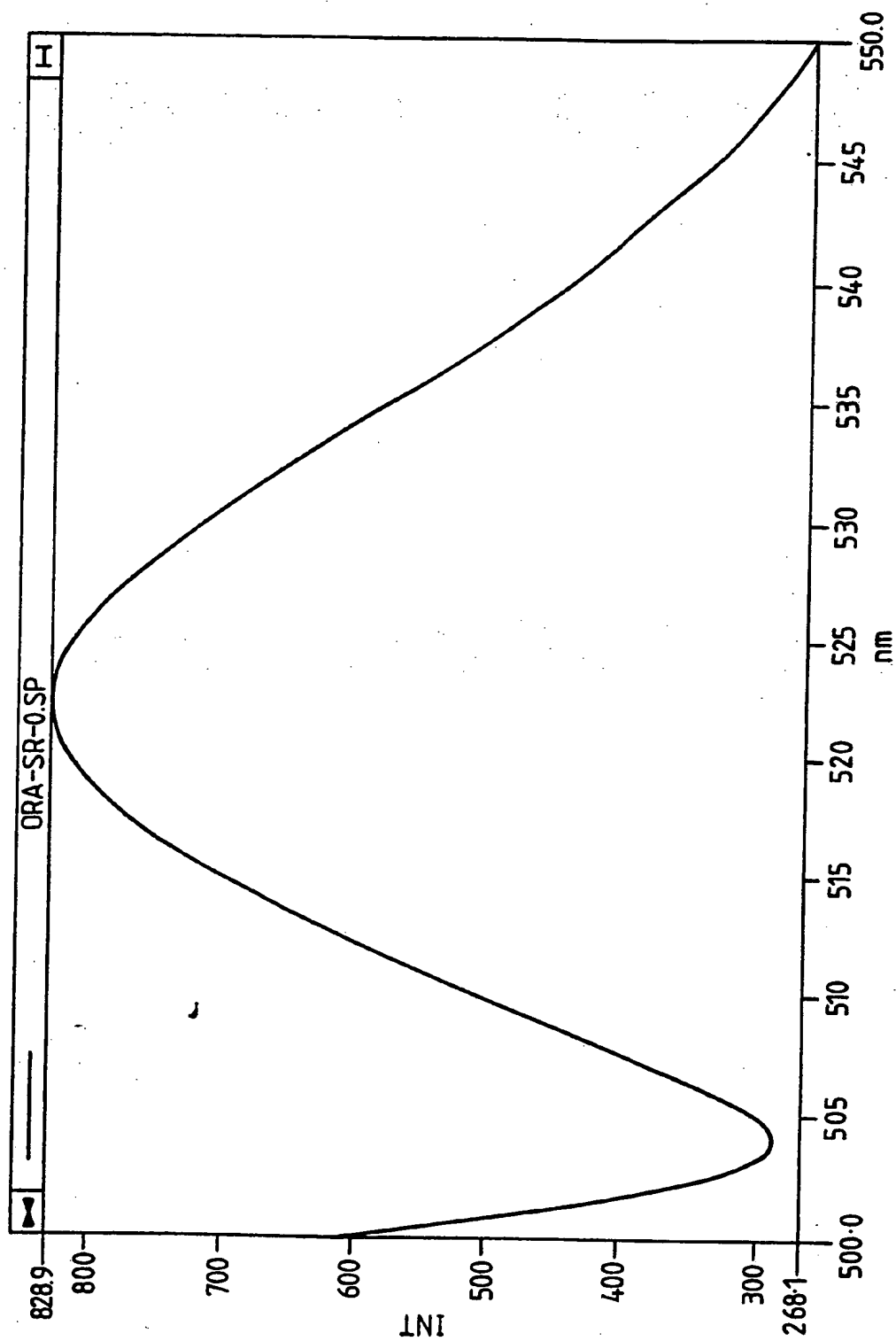


FIG. 2b



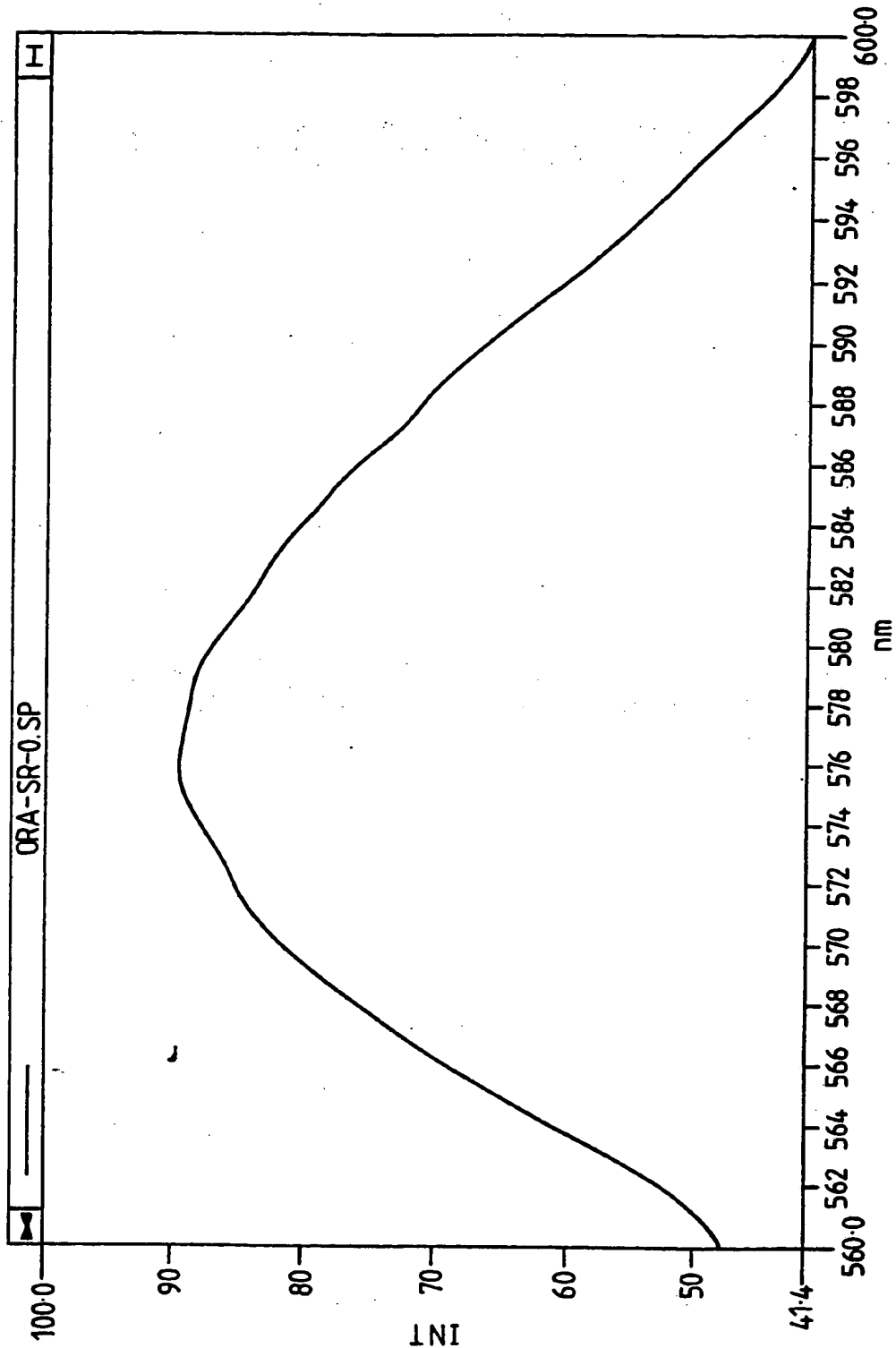
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FIG. 3a



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FIG. 3b



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FIG. 4

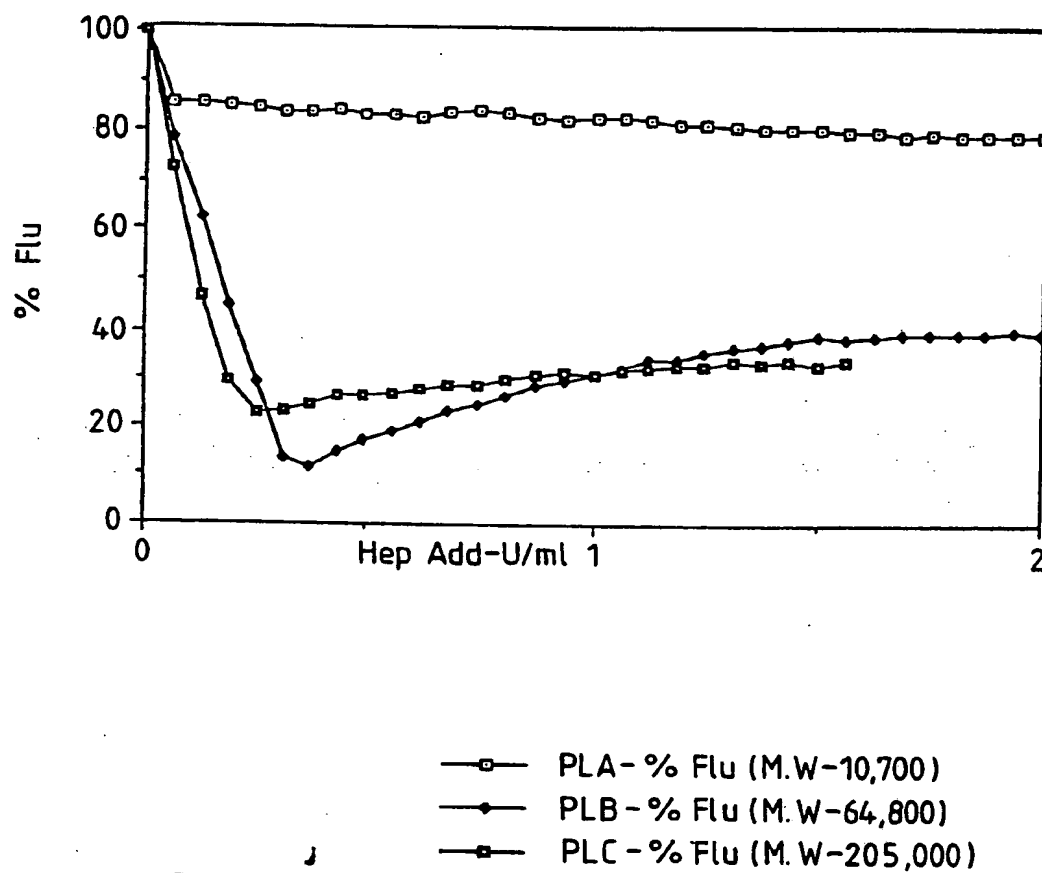




FIG. 5 6/7

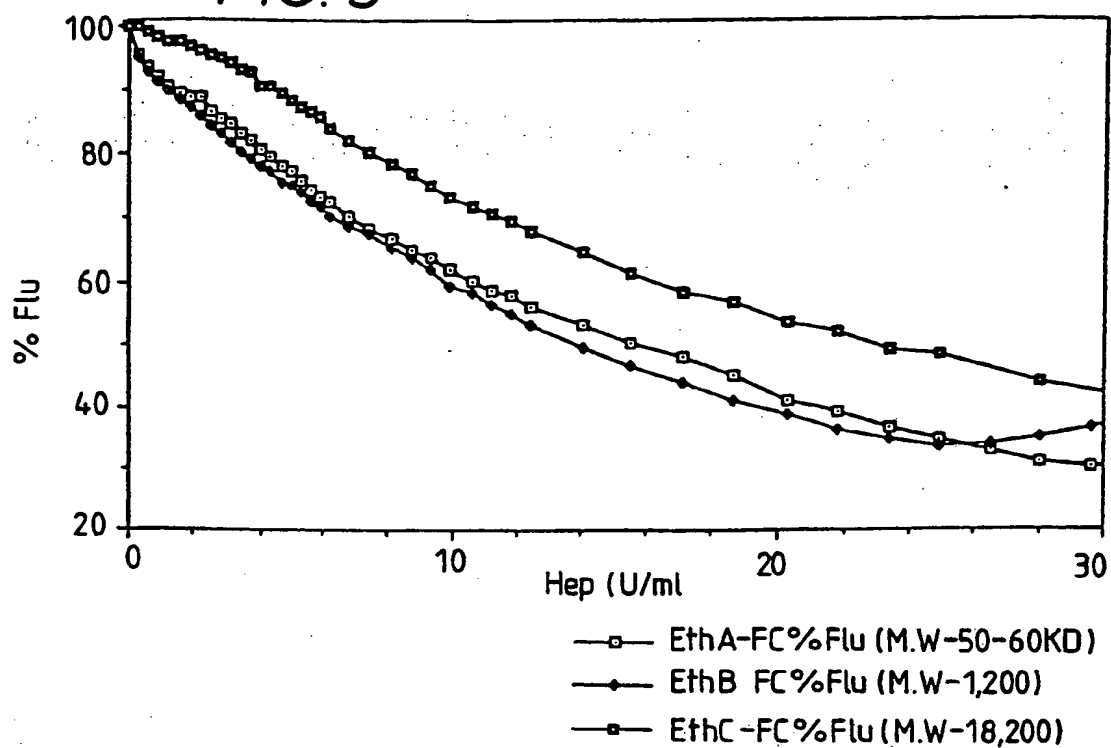


FIG. 6

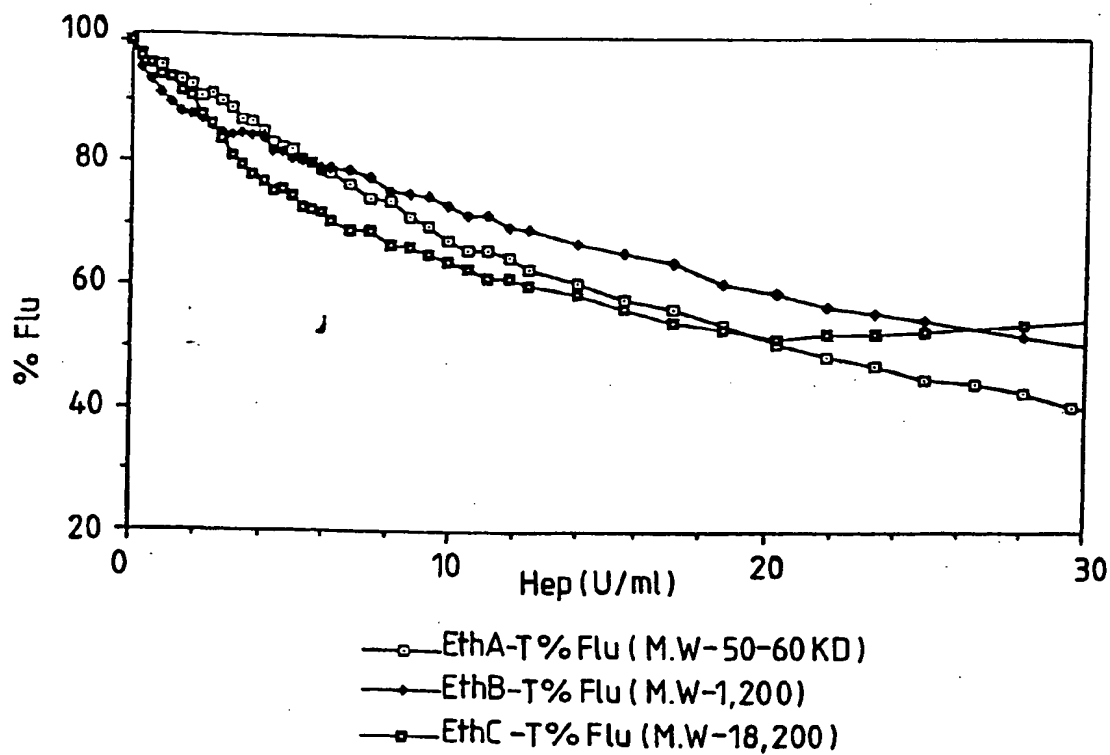


FIG. 7

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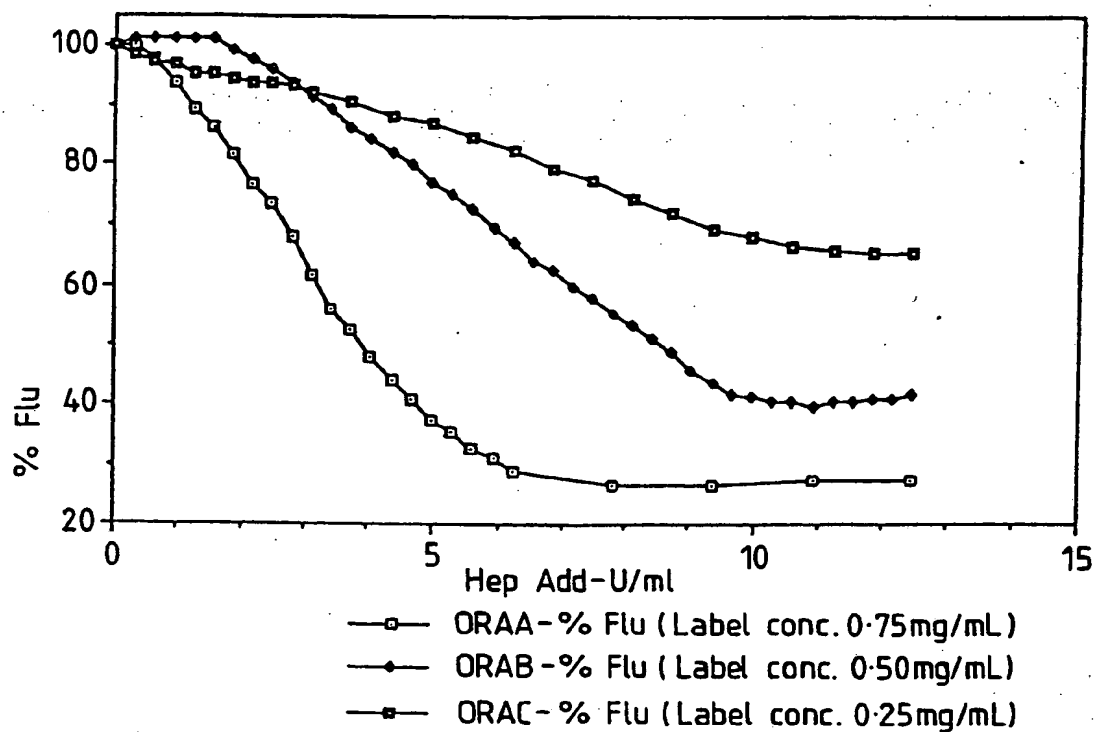
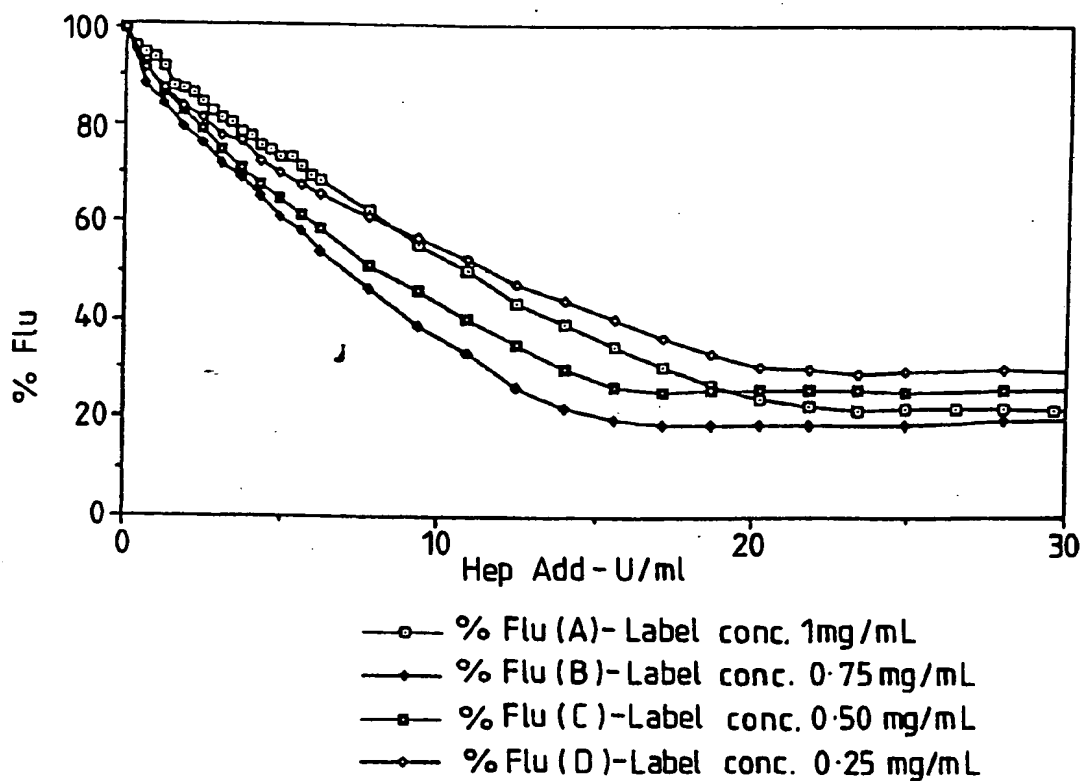


FIG. 8



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/00197

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 G01N33/66; G01N33/86		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	G01N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	DE,A,4 020 966 (HOECHST AG) 16 January 1992 see claims 1-3	1-18
Y	EP,A,0 362 077 (SANOFI) 4 April 1990 cited in the application see the whole document	1-18
A	FR,A,2 640 385 (SERBIO) 15 June 1990	
A	PATENT ABSTRACTS OF JAPAN vol. 3, no. 104 (C-57)4 September 1979 & JP,A,54 083 095 ( KUNIHICO TAKAGI ) 7 February 1979 see abstract	
	-/--	
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
07 MAY 1993	01.06.93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	CARTAGENA ABELLA P	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 105, no. 17, 27 October 1986, Columbus, Ohio, US; abstract no. 145902s, G. R. JONES ET AL. 'A COMPARISON OF THE STRENGTH OF BINDING OF ANTITROMBIN III, PROTEMINE AND POLY(L-LYSINE) TO HEPARIN SAMPLES OF DIFFERENT ANTICOAGULANT ACTIVITIES.' page 41-42 ; see abstract & BIOCHIM. BIOPHYS. ACTA vol. 883, no. 1, 1986, pages 69 - 76 -----	
A	EP,A,0 281 128 (SLOVENSKA AKADEMIA VIED) 7 September 1988 -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9300197  
SA 69535

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-4020966	16-01-92	None	
EP-A-0362077	04-04-90	FR-A- 2636967	30-03-90
		AU-B- 631144	19-11-92
		AU-A- 4241989	05-04-90
		JP-A- 2150297	08-06-90
FR-A-2640385	15-06-90	None	
EP-A-0281128	07-09-88	None	

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